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Survey of human immunodeficiency virus (HIV)-seropositive patients with mycobacterial infection in Japan

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Abstract *Objective*. To assess DNA polymorphisms in mycobacterial isolates obtained from human immunodeficiency virus (HIV)-seropositive patients with tuberculosis in Japan from 1996 to 2003.

Methods. Restriction fragment length polymorphisms (RFLP) from *Mycobacterium tuberculosis* and *Mycobacterium avium* isolates obtained from individual seropositive patients with tuberculosis ($n=78$) were analysed with the use of IS*6110* and $(CGG)_5$ or IS*1245* and IS*1311*, respectively, as markers. As a control, the same procedures were applied to isolates from HIV-seronegative tuberculosis patients ($n=87$).

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Results. Of 86 mycobacterial strains, *M. tuberculosis*, *M. avium* and *Mycobacterium chelonae* were identified in 48 (55.8%), 36 (41.9%) and 2 (2.3%) isolates, respectively. The obtained RFLP patterns of *M. tuberculosis* isolates from both the HIV-seropositive and -seronegative groups were variable, suggesting no obvious clustering among the isolates. Similar results were obtained in isolates of *M. avium*.

Conclusions. This is the first report on the molecular epidemiology of *Mycobacterium* spp. isolated from HIV-seropositive patients in Japan. The results indicate that no particular clones of *M. tuberculosis* or *M. avium* prevail in HIV-seropositive patients in Japan. Further monitoring of mycobacterial infection associated with HIV infection in Japan should be continued.

Introduction

In recent years, a decline in the number of new patients with acquired immunodeficiency syndrome (AIDS) has been observed in several industrialized countries, including the United States, Western European countries, Australia and New Zealand.[1-6] However, no decline in patients with human immunodeficiency virus (HIV) has been observed in Japan.[7] Mycobacterial infections, such as those of *Mycobacterium tuberculosis* and *Mycobacterium avium*, are important opportunistic infections in HIV-seropositive patients. With respect to tuberculosis (TB), several studies based on clinical observations[8-12] and on epidemiologic surveys[13-17] have provided evidence that HIV infection is a risk factor for the development of active and often lethal TB. Outbreaks of TB among communities of HIV patients have been reported in the United States, but multidrug resistant (MDR) *M. tuberculosis* strains were rarely isolated from these patients.[12,18] In sub-Saharan Africa, TB associated with HIV has played an important role in increasing TB transmission throughout the population.[17,19]

Non-tuberculous mycobacterial infection can be difficult to treat because of primary resistance against most of the commonly used anti-tubercular drugs, such as isoniazid, rifampin, streptomycin, ethambutol, pyrazinamide and kanamycin.[20] A relatively high prevalence of non-tuberculous mycobacterial infections has been observed in HIV/AIDS patients, and 25-50% of patients with AIDS in the United States and Europe are infected with this group of bacteria, primarily with *M. avium*, which mainly causes disseminated mycobacteraemia in AIDS patients.[21]

Japan is considered to have a low prevalence of HIV/AIDS, with a cumulative number of 2556 AIDS cases and 5140 HIV cases reported by the end of 2002.[7] However, the recent trend of HIV cases shows a substantial increase, particularly among men who have sex with men and youth/young adults. A considerable number of HIV patients in Japan have experienced discrimination or breach of confidentiality and they feel insufficiency of social and economical supports.[22] Patients with mycobacterial infection used to be discriminated, but the prejudice toward the patients declines. The medical, social and economic backgrounds of HIV patients in Japan differ considerably from those in regions such as North America, Europe and Africa. The correlation between HIV and mycobacterial infections in Japan may also differ from that in countries where research on AIDS-related diseases is well developed. Survey of the occurrence and clinical profiles of these infections is important for the development of countermeasures against mycobacteria and HIV coinfection. In this study, we analysed the current prevalence, clinical features and epidemiologic findings of mycobacterial infection associated with HIV infection in Japan.

Materials and methods

Bacterial isolates and clinical data

From 1996 to 2003, 86 clinical mycobacterial isolates were obtained from eight hospitals in Japan: the International Medical Centre of Japan (IMCJ) (Tokyo); Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association (JATA) (Tokyo); National Tokyo Hospital (Tokyo); Tokyo Metropolitan Komagome Hospital (Tokyo); Social Insurance Central General Hospital (Tokyo); National Nishi-Kofu Hospital (Yamanashi); National Osaka National Hospital (Osaka) and National Kyushu Medical Centre (Fukuoka). Clinical information on individual patients was obtained by the physicians in charge with questionnaire on mycobacterial isolation date, history of previous mycobacterial infection, microscopic observation of

Table 1 Nationality and sex of HIV-positive patients with mycobacterial infection in Japan

Nationality	No. of patients	Male:female	Mycobacteria species
Japanese	33	31:2	*M. tuberculosis*: 21 *M. avium*: 11 *M. chelonae*: 1
Non-Japanese	16	9:7	*M. tuberculosis*: 9 *M. avium*: 6 *M. chelonae*: 1
Unknown[a]	37	33:2, unknown[a]:2	*M. tuberculosis*: 18 *M. avium*: 19 *M. chelonae*: 0
Total	86	74:10, unknown[a]:2	*M. tuberculosis*: 48 *M. avium*: 36 *M. chelonae*: 2

[a] Nationality or sex of these patients was not disclosed due to the ethics code of the corresponding hospital.

sputa, sites of infection (pulmonary or extrapulmonary), peripheral blood $CD4^+$ lymphocyte number, chemotherapeutic regimens and standard demographic data. The Ethics Committees in each hospital approved this study (IMCJ-H13-54) and all patients gave a written informed consent.

As a control for *M. tuberculosis* genotyping, 87 clinical isolates from adult HIV-seronegative tuberculosis patients without any serious complication at IMCJ were used. Since other hospitals, except JATA, have no ward for TB patients and the RFLP patterns of *M. tuberculosis* isolates from JATA and IMCJ were variable, and showed no obvious clustering among the isolates.

Mycobacterial culture and identification of strains

Bacteria were grown on egg-based Ogawa medium (Kyokuto Pharmaceutical Co., Ltd, Tokyo, Japan) for 3-5 weeks. Cultured organisms were applied to a polymerase chain reaction (PCR) kit for *M. tuberculosis* diagnosis (Amplicor *Mycobacterium tuberculosis* Test, Roche Diagnostic Systems, Inc., Branchburg, NJ), and PCR-negative organisms were further applied to an identification kit for mycobacterial species that uses DNA-DNA hybridization (DDH Mycobacteria, Kyokuto Pharmaceutical Co., Ltd).

Drug sensitivity testing

Drug sensitivity of *M. tuberculosis* strains was tested by two agar proportion methods, one with Middlebrook 7H10 agar medium, as recommended by the U.S. Public Health Service,[23] and the other with egg-based Ogawa medium, as recommended by the Japanese Society for Tuberculosis (Vit Spectrum-SR™, Kyokuto Pharmaceutical Co., Ltd).

DNA fingerprinting

Chromosomal DNA from mycobacterial isolates was prepared as described previously[24,25] but with slight modification. The DNA was precipitated in isopropanol, and the precipitates were redissolved in 20 μl 0.1X TE buffer.

For IS6110- and $(CGG)_5$-restriction fragment length polymorphisms (RFLP)[26] of *M. tuberculosis*, DNA was digested overnight with restriction enzymes *Pvu*II and *Alu*I (Takara Bio, Inc., Shiga, Japan), respectively. The digested fragments were separated by electrophoresis on 1% agarose gels. A 1-kb DNA ladder (Promega Corp., Madison, WI) was used as a marker. The agarose gels were stained with ethidium bromide, and the results were recorded photographically. DNA fragments were transferred onto N^+ Hybond membrane (Amersham Biosciences, Little Chalfont, UK), and the DNA was fixed to the membrane by UV illumination. The IS*6110* probe was a 245-bp DNA fragment amplified by PCR as described previously.[25] The 15-mer oligonucleotide $(CGG)_5$ was synthesized by Nippn Techno Cluster, Inc., Tokyo, Japan. The probes were labelled with horseradish peroxidase by the ECL Direct™ System (Amersham Biosciences). Hybridization was conducted with the ECL Direct™ System, according to the recommendations of the manufacturer. Autoradiographs were obtained by exposing the membranes to X-ray film.

For IS*1245*-[27] and IS*1311*-RFLP[28] of *M. avium*, DNA was digested overnight with *Pvu*II. The IS*1245* and IS*1311* probes were 427 and 200-bp DNA fragments, respectively, and were amplified by

PCR as described previously.[27,28] Briefly, the oligonucleotides for IS*1245*, 5′-GCCGCCGAAACGATCTAC-3′ and 5′-AGGTGGCGTCGAGGAAGAC-3′,[27] and for IS*1311*, 5′-GTCGGGTTGGGCGAAGAT-3′ and 5′-GTGCAGCTGGTGATCTCTGA-3′,[28] were used to amplify the fragments prepared from purified chromosomal DNA from *M. avium* ATCC 25291 by PCR.

Analysis

Fingerprinting patterns of *M. tuberculosis* or *M. avium* were analysed with Molecular Analyst Fingerprinting Plus Software, version 1.6 (Bio-Rad Laboratories, Inc., Hercules, CA). To facilitate comparison of the fingerprinting patterns, normalization was performed relative to the molecular-weight markers. Each dendrogram was calculated according to the unweighted-pair group method with average linkage according to the supplier's instructions.

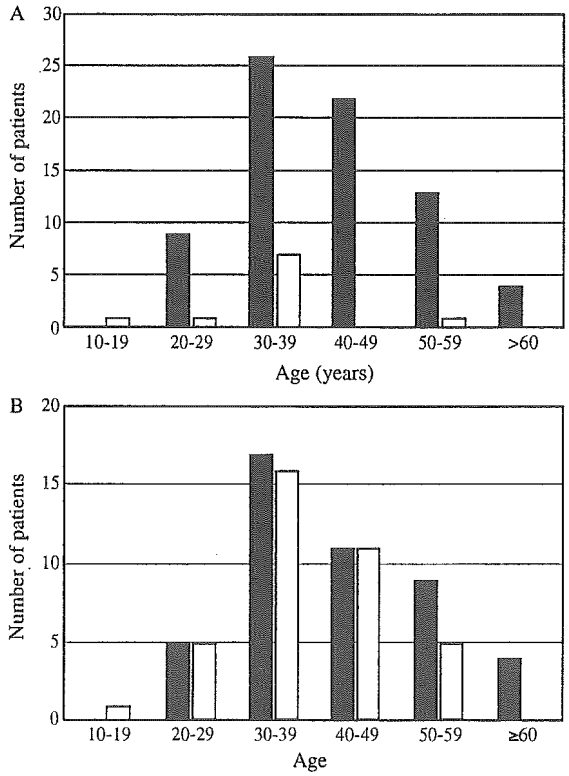

Figure 1 Distribution of 84 mycobacterial infections in HIV-seropositive patients. Panel A: age (years) and sex distribution. Filled bars, male; open bars, female. Panel B: age (years) and pathogenic agent distribution. Filled bars, tuberculosis patients; open bars, non-tuberculous mycobacterial-infected patients.

Results

Mycobacterial infection in HIV-seropositive patients

From 86 HIV-seropositive patients, 48 (55.8%) *M. tuberculosis*, 36 (41.9%) *M. avium*, and 2 (2.3%) *Mycobacterium chelonae* isolates were identified (Table 1).

Nationality and sex are also listed in Table 1. Mean age was 40.5±12.2 years, ranging from 11 to 68 years. Most mycobacteria and HIV coinfected patients were aged 30-39 years (Fig. 1). The most frequent route of HIV infection was sexual transmission (90%); other routes were infection by blood products (5%), drug abuse (5%), mother-to-child infection (1%) and unknown (1%). With respect to mycobacterial infection, 48 and three individuals had primary and recurrent infection, respectively. There was no corresponding record for the remaining patients.

Profile of HIV-seropositive patients with *M. tuberculosis*

In 46 of the 48 tubeculosis patients, the ratio of males/females was 43/3 (Table 1). Mean age was 42.7±11.9 years, ranging from 22 to 68 years. Twenty-five patients had combined pulmonary and extra-pulmonary infection, mainly due to miliary tuberculosis. A total of 56.3% of the 48 patients had pulmonary tuberculosis, as evidenced by positive microscopy smears. Peripheral blood $CD4^+$ cell counts at the time of TB diagnosis ranged from 6 to 331/mm^3, and the median was 62/mm^3.

According to drug sensitivity testing, 43 isolates (89.6%) were sensitive to anti-tubercular drugs, 3 (6.3%) were resistant to a single drug, and 2 (4.2%) were resistant to 2 and 5 drugs, respectively.

In 87 HIV-seronegative TB patients with tuberculosis, 82 were Japanese and five were non-Japanese. The ratio of males/females was 56/31. Mean age was 53.3±20.5 years (56.1±19.0 years for males and 48.6±22.0 years for females), ranging from 18 to 95 years (18-90 for males and 18-95 for females) and patients over 40 years of age accounted for 66.7% of the total. According to drug sensitivity testing, 75 isolates (86.2%) were sensitive to anti-tubercular drugs, 6 (6.9%) were resistant to a single drug, and 6 (6.9%) were resistant to 2 and 6 drugs, respectively.

RFLP analysis of *M. tuberculosis*

To determine whether specific strain(s) of tubercular bacilli prevail among HIV-seropositive patients in Japan, we analysed DNA fingerprints of the isolates by RFLP analysis. Thirty-three of the 48 *M. tuberculosis* clinical isolates were analysed by RFLP, and the patterns are shown in Fig. 2.

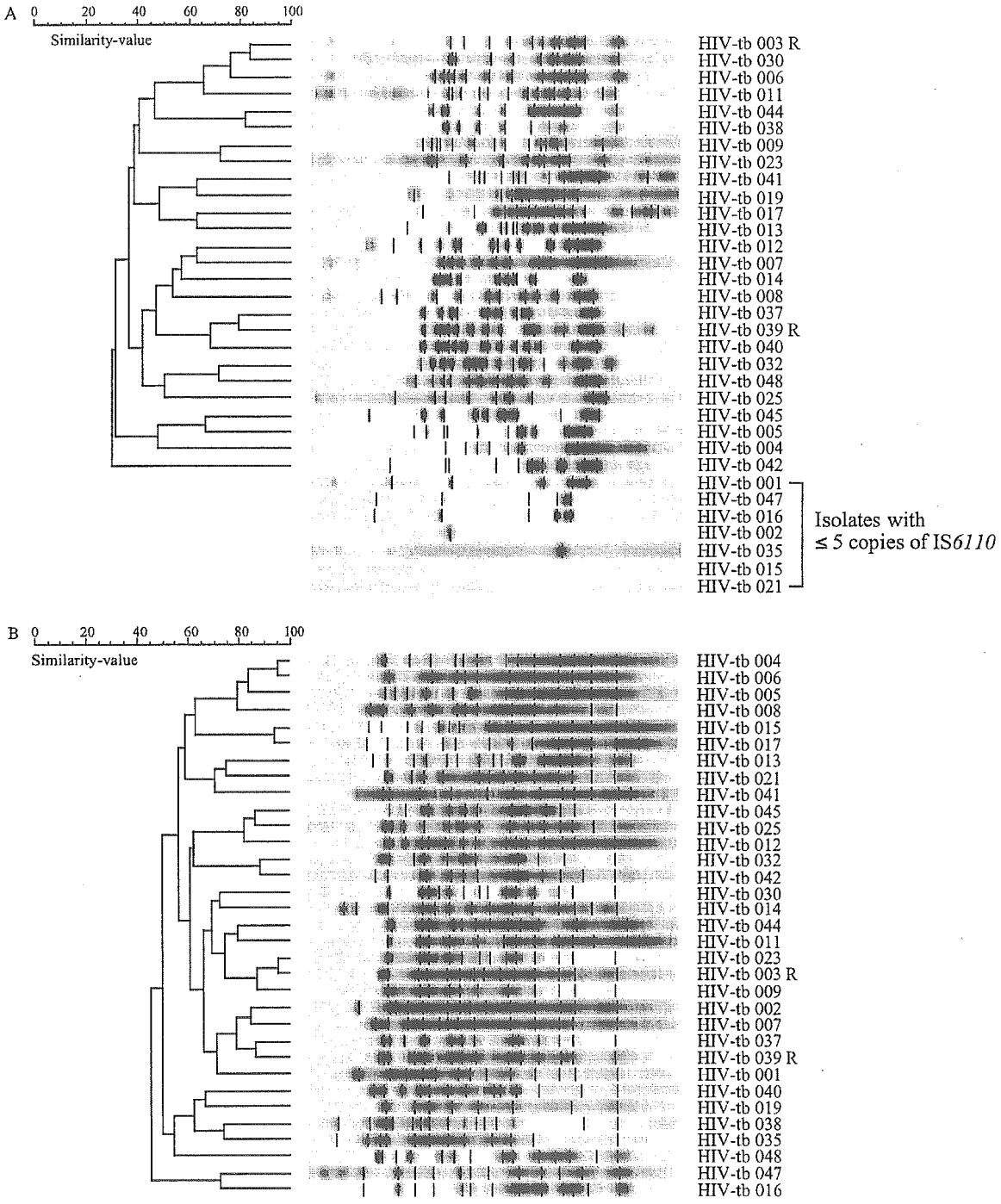

Figure 2 IS*6110*- and $(CGG)_5$-probed DNA fingerprinting patterns of *M. tuberculosis* clinical isolates from HIV-seropositive patients and corresponding dendrograms. The fingerprint patterns are ordered by similarity. The corresponding dendrograms are to the left of the patterns. The position of each IS*6110* (A) or $(CGG)_5$ (B) band is normalized so that the patterns for all strains are comparable. In the IS*6110*-probed DNA fingerprint patterns, isolates with five or fewer copies are indicated in Panel A. The isolates are named as follows: a prefix of 'HIV-tb' indicates an HIV-seropositive patient-derived isolate, and a suffix of 'R' indicates a drug-resistant isolate. For example, HIV-tb 003 R is an HIV-seropositive patient-derived isolate.

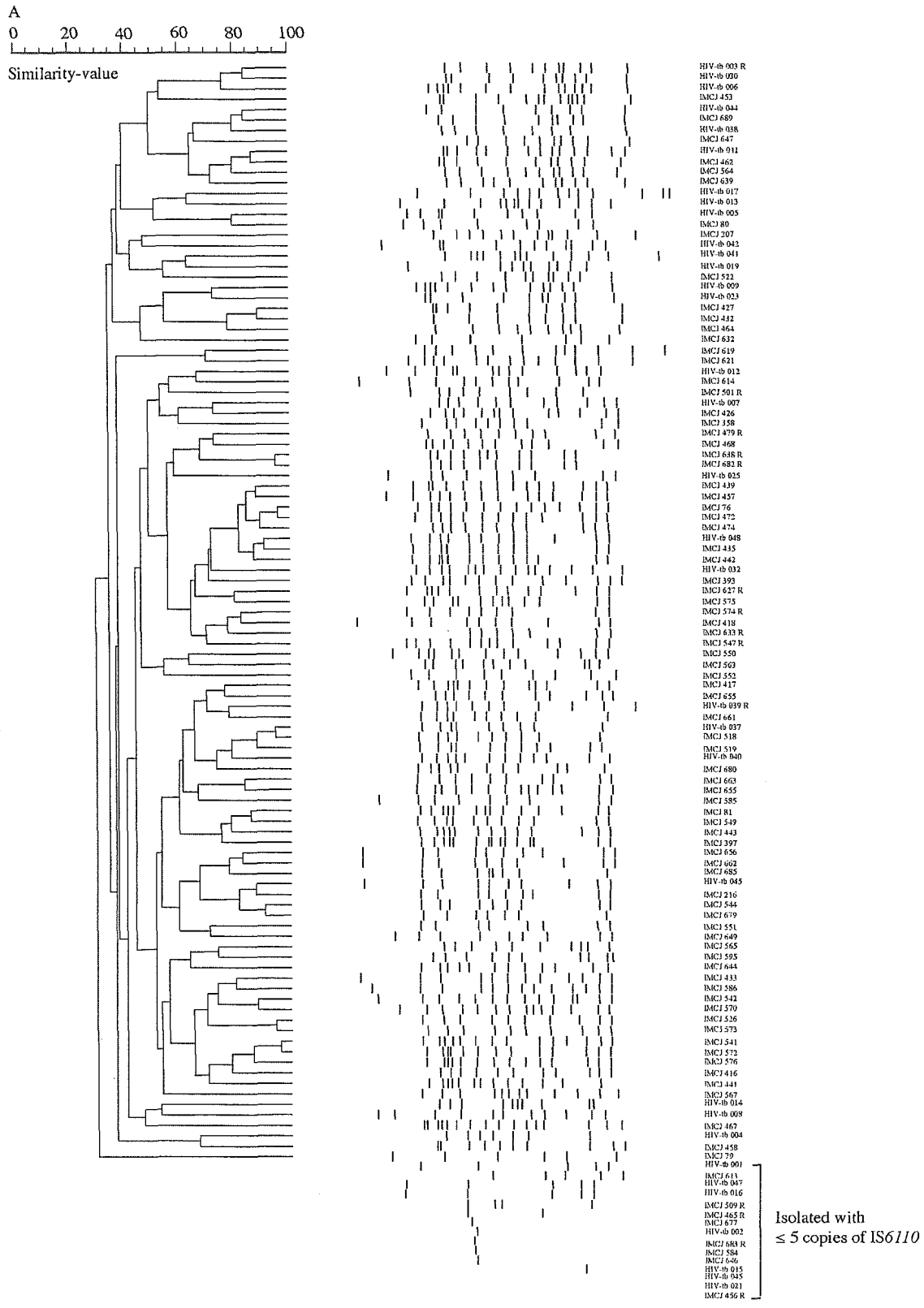

Figure 3. IS*6110*- and $(CGG)_5$-probed DNA fingerprinting patterns of *M. tuberculosis* clinical isolates from HIV-seropositive and HIV-seronegative patients and corresponding dendrograms. The fingerprint patterns are ordered by similarity. The corresponding dendrograms are to the left of the patterns. The position of each IS*6110* (A) or $(CGG)_5$ (B) band is normalized so that the patterns for all strains are comparable. In the IS*6110*-probed DNA fingerprint patterns, isolates with five or fewer copies are indicated in Panel A. The isolates are named as follows: a prefix of 'HIV-tb' indicates an HIV-seropositive patient-derived isolate, a prefix of 'IMCJ' indicates an HIV-seronegative patient-derived isolate, and a suffix of 'R' indicates a drug-resistant isolate. For example, IMCJ 627 R is an HIV-seronegative patient-derived isolate.

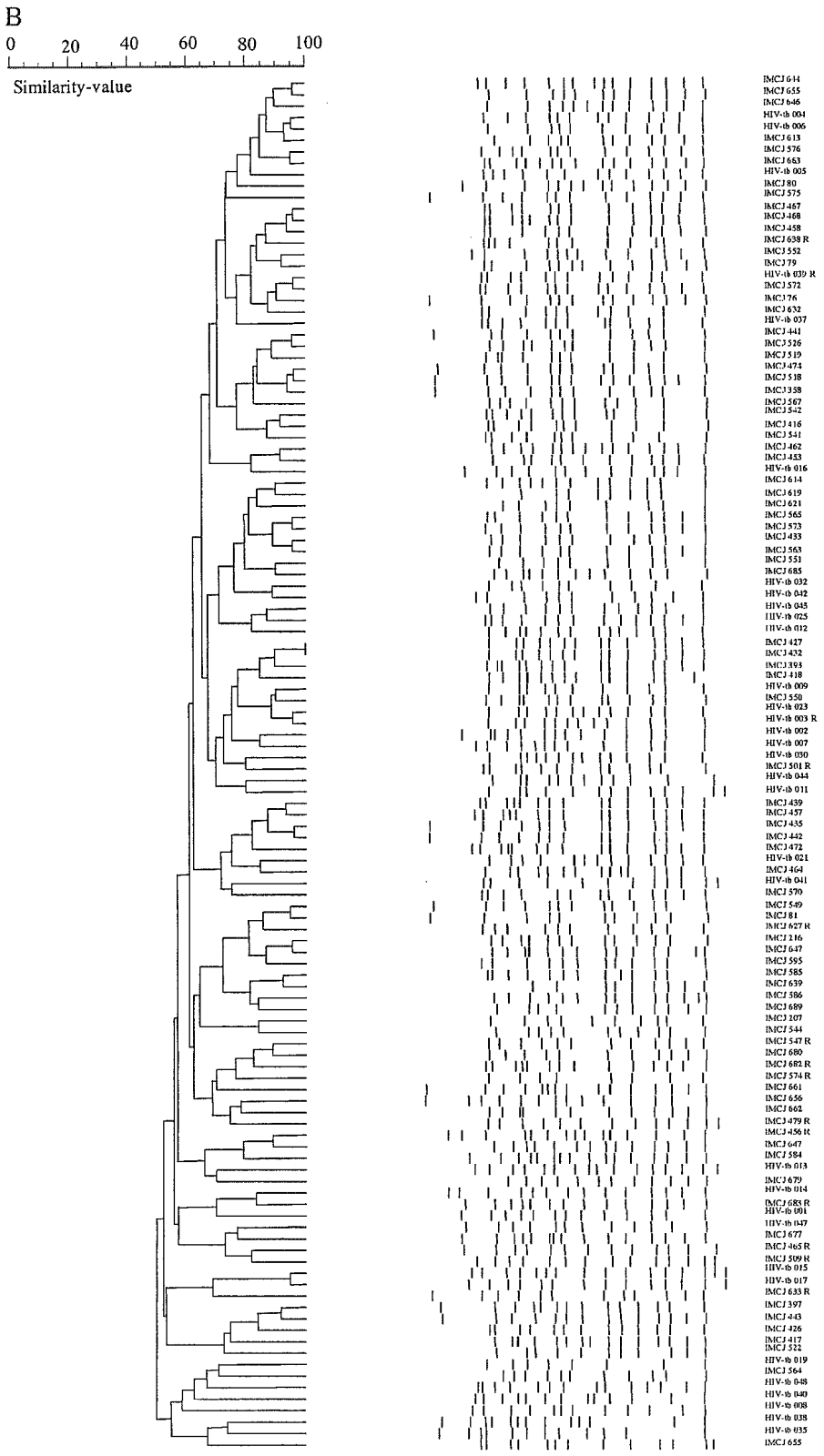

Figure 3 (*continued*)

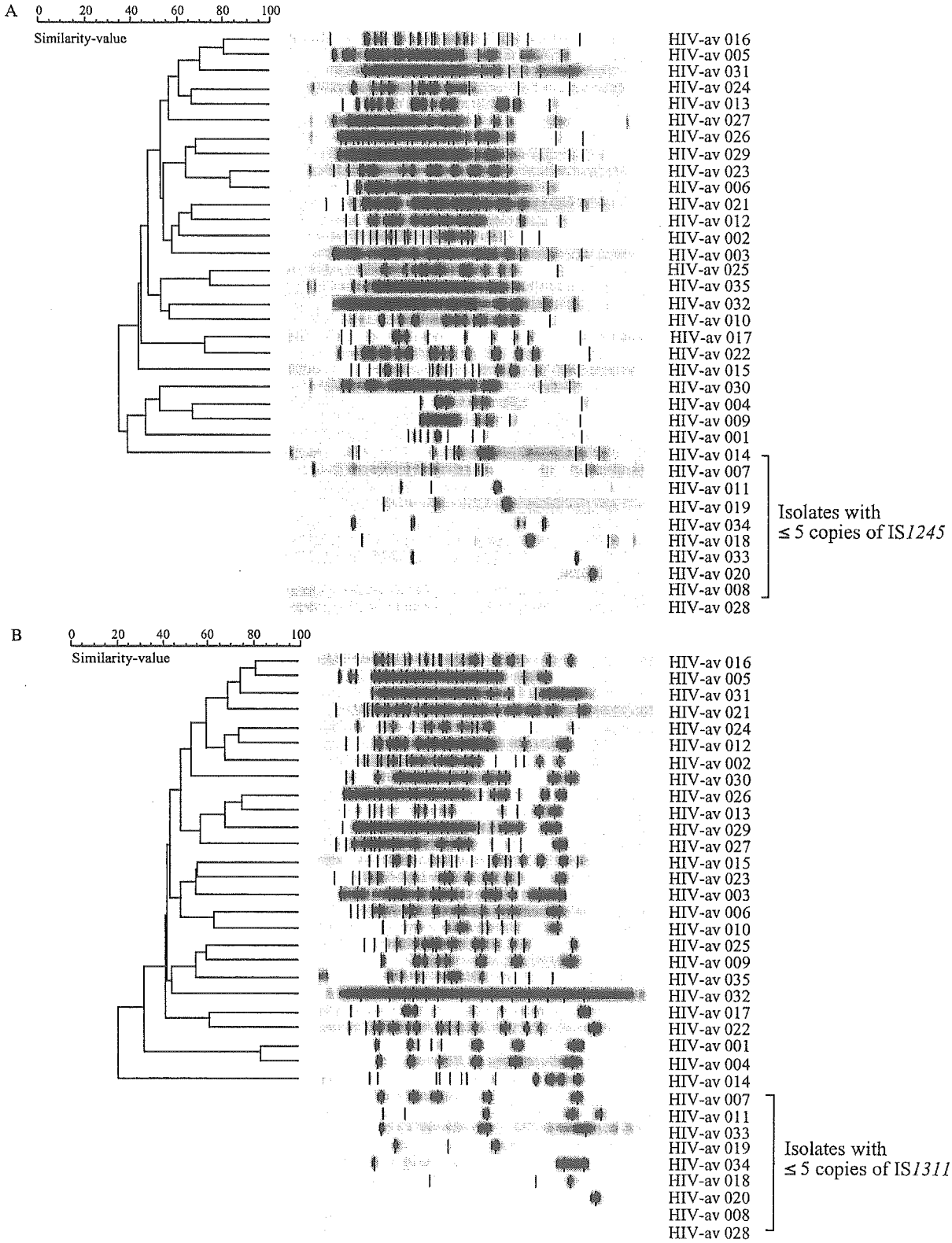

Figure 4 IS*1245*- and IS*1311*-probed DNA fingerprinting patterns of *M. avium* clinical isolates from HIV-seropositive patients in Japan and corresponding dendrograms. The fingerprint patterns are ordered by similarity. The corresponding dendrograms are to the left of the patterns. The position of each IS*1245* (A) or IS*1311* (B) band is normalized so that the patterns for all strains are comparable. In both the IS*1245*- and IS*1311*-probed DNA fingerprint patterns, isolates with five or fewer copies are indicated. The isolates are named as follows: a prefix of 'HIV-av' indicates an HIV-seropositive patient-derived isolate.

The number of IS*6110* bands in the isolates ranged from 0 to 15 (Fig. 2A). Thirty-one different IS*6110* fingerprinting patterns were observed in the isolates. Seven isolates (21.2%) showed 0-5 IS*6110* bands, these isolates were insufficient in band number for cluster analysis. Identical patterns were not found among the isolates (Fig. 2A).

The number of $(CGG)_5$ bands of the copy isolates ranged from 8 to 16 (Fig. 2B). Thirty-three different $(CGG)_5$ fingerprinting patterns were observed in the isolates. Identical patterns were not found among the isolates (Fig. 2B). Three pairs of isolates (HIV-tb 004 and HIV-tb 006, HIV-tb 015 and HIV-tb 017 and HIV-tb 03 and HIV-tb 023) were closely related, with 90% or more similarity. However, the corresponding patients did not show any linkage such as hospital or date of sample isolation.

Comparison of RFLP patterns between HIV-seropositive and -seronegative TB patients

To assess whether the same kinds of mycobacteria prevail in HIV-seropositive and -seronegative patients, RFLP patterns of *M. tuberculosis* clinical isolates were investigated in both groups. In IS*6110*- or $(CGG)_5$-patterns from both groups, the patterns from both HIV-seropositive and -seronegative patients did not consist of apparent clusters and appeared to segregate randomly in the dendrograms (Fig. 3).

Profile of HIV-seropositive patients with *M. avium* infection

The number of HIV-seropositive patients with *M. avium* infection was 36 (Table 1). Mean age was 37.4 ± 9.9 years, ranging from 11 to 56 years. Most of the *M. avium*-infected patients (58.3%) suffered from disseminated infection, and the sputa of 88.9% patients were culture-positive but smear-negative upon preliminary mycobacterial examination. Almost all the *M. avium* isolates were resistant to all anti-TB drugs. Peripheral blood $CD4^+$ cell counts of 34 patients (unknown: 2) at the time of *M. avium* diagnosis ranged from 0 to 202/mm^3, and the mean $CD4^+$ cell count was 38.6 ± 60.4/mm^3. In 26 of 34 patients (76.4%), the $CD4^+$ cell counts were less than 50/mm^3.

RFLP analysis of *M. avium*

The RFLP patterns of 35 of 36 *M. avium* isolates were investigated (Fig. 4). The number of IS*1245*- and IS*1311*-bands ranged from 0 to 25 and from 0 to 23, respectively, and analysis showed 33 different patterns of each. Nine isolates (25.7%) showed 0-5 bands; these isolates were insufficient for cluster analysis because of few numbers of IS*1245* or IS*1311* bands. Among the isolates, identical patterns were not found. Cluster analysis revealed no clusters. These results indicate that no particular strain of *M. avium* prevailed among HIV-seropositive patients.

Discussion

We analysed mycobacterial isolates obtained from HIV-seropositive patients and found that *M. tuberculosis* and *M. avium* accounted for a large proportion of HIV-associated mycobacterial infection in Japan. Although *Mycobacterium kansasii* is also known to be associated with AIDS,[29,30] it was not isolated in this study. Two isolates of *M. chelonae* were obtained from stool specimens of patients.

It has been suggested that recurrent TB is responsible for most cases of HIV-associated TB, particularly in countries with high-level of transmission.[31] Kanazawa et al.[32] reported that the majority of HIV-positive Japanese patients with TB (83%) were more than 40 years of age and had recurrent TB. In the present study, the age of HIV-seropositive patients shifted to the 30s, suggesting that TB incidence among HIV-positive patients in Japan is transforming from recurrence in older persons to primary infection in younger persons.

With respect to drug resistance, 10.4% of the strains obtained from HIV-seropositive patients showed resistance to one or more anti-TB drugs. Abe et al.[33] reported that 10.3% of *M. tuberculosis* isolates from patients in Japan were resistant to one or more of the four first-line anti-TB drugs: isoniazid, rifampin, streptomycin and ethambutol. A 1996 report noted that the drug resistance rate in New York City was 33%.[34]

We found that both the IS*6110* and $(CGG)_5$ fingerprinting patterns of *M. tuberculosis* isolates from HIV-seropositive patients in Japan differed from those of a TB outbreak in New York City[12,18] and of isolates from the patients in Lima, Peru.[16] Comparing RFLP patterns of *M. tuberculosis* isolates from HIV-seropositive patients with those from HIV-seronegative patients, we found that the DNA fingerprints did not distinguish between these two TB patient groups. These data indicate that TB transmission in Japan occurs via HIV-seronegative TB patients rather than via HIV-seropositive TB patients. The epidemiological studies in Botswana[17] and Tanzania[19] showed no clustering any particular

pattern of DNA fingerprints. These findings are consistent with our present results.

Patients infected with *M. avium* suffer from chronic lung disease. In patients with HIV-associated *M. avium* infection, it is thought that pulmonary symptoms will develop when $CD4^+$ lymphocyte counts fall below $100/mm^3$. The median $CD4^+$ lymphocyte count at *M. avium* diagnosis was $10/mm^3$, and at that time the majority of patients showed disseminated *M. avium* infection. Almost all *M. avium*-infected patients in the present study were in advanced stages of AIDS. *M. avium* organisms can be isolated from environmental sources such as water or soil.[35-37] Because they are capable of causing infection in animals, e.g. birds and pigs, it has been postulated that the source of human infection is either the environment or from animals. Ichiyama et al.[38] searched sources of soil, water and dust in Japan and found *M. avium* isolates in 68.0% of dust samples tested. It is believed that the most frequent mode of *M. avium* infection in humans occurs by inhalation or by deglutition of the agent from environmental sources.[37,39,40] To prevent infection with this agent in HIV-seropositive patients, further studies are needed to identify original sources and to further elucidate infectious routes.

In conclusion, the number of HIV patients in Japan is increasing; according to the latest report,[7] the number is over 10 000. The number of TB patients in Japan remains higher than in other developed countries.[41] However, the number of HIV-infected patients with mycobacterial infection in Japan is limited. With respect to TB, no outbreak among HIV-seropositive patients was found. Further monitoring of mycobacterial infection associated with HIV infection in Japan should be continued.

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Cloning and Characterization of a Novel Trimethoprim-Resistant Dihydrofolate Reductase from a Nosocomial Isolate of *Staphylococcus aureus* CM.S2 (IMCJ1454)

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A novel gene, *dfrG*, encoding a trimethoprim (TMP)-resistant dihydrofolate reductase (DHFR, designated S3DHFR) was cloned from a clinical isolate of methicillin-resistant *Staphylococcus aureus*. *Escherichia coli* expressing *dfrG* was highly resistant to TMP. Recombinant S3DHFR exhibited DHFR activity that was not inhibited by TMP.

Trimethoprim (TMP) is a potent inhibitor of bacterial dihydrofolate reductase (DHFR) and is effective in vitro against methicillin-resistant *Staphylococcus aureus* (MRSA). In combination with sulfamethoxazole, TMP has been used successfully to treat patients infected with MRSA and is effective at eradicating carriage (10, 16). Resistance of *S. aureus* to TMP was first reported in the 1980s (12) and was found to be due to plasmid-mediated production of an additional DHFR that was less sensitive to TMP than intrinsic DFHR (*S. aureus* DHFR [SaDHFR]) encoded by the *dfrB* gene on the chromosome (1, 12). Plasmid-mediated production of an additional TMP-resistant DHFR is one of the most common mechanisms of resistance to TMP in bacterial organisms. At least 14 different types of TMP-resistant DHFRs in gram-negative bacteria have been reported (10); however, only a limited number of TMP-resistant DHFRs in gram-positive bacteria have been reported (10).

A total of 43 clinical isolates of MRSA from Chiang Mai, Thailand, and 244 clinical isolates of MRSA from Tokyo, Japan, were analyzed in this study. All isolates were positive for *dfrB* by PCR and also positive for *femB* encoding coagulase and for *mecA* associated with methicillin resistance. All isolates from Chiang Mai, Thailand, were resistant to TMP, whereas all those from Tokyo, Japan, except one, *S. aureus* IMCJ934, were sensitive to TMP (Table 1). Crude extracts prepared from a TMP-resistant isolate from Chiang Mai, *S. aureus* CM.S2 (IMCJ1454), showed DHFR activity, and K_m values of the extract for DHF and NADPH were similar to those of crude extracts from TMP-sensitive strain ATCC 25923 (Table 2); however, the 50% inhibitory concentration (IC_{50}) of TMP for the crude extract of strain CM.S2 was more than 15,000-fold greater than that of ATCC 25923.

HindIII-digested fragments of the *S. aureus* CM.S2 genome were cloned, transformed into *Escherichia coli* DH5α cells, and selected on agar medium containing TMP (8 μg/ml). The resultant plasmid, named pSA1, had a 3.5-kb insert containing a complete open reading frame (ORF) surrounded by truncated ORFs (data not shown). The complete ORF consisted of 498 bp encoding a putative protein of 165 amino acids with similarities to TMP-resistant DHFR from *Staphylococcus haemolyticus* (79% identity) (7), *Bacillus anthracis* (67% identity) (2), and *Bacillus cereus* (65% identity) (15) (Fig. 1). The deduced

TABLE 1. MICs of trimethoprim in *S. aureus* and *E. coli* strains

Strain	MIC of TMP (μg/ml)	Characteristic(s) or genotype
S. aureus CM.S2 (IMCJ1454)	>512	Clinical isolate from Chiang Mai, Thailand, in 2003
S. aureus IMCJ934	>512	Clinical isolate from Tokyo, Japan, in 2001
S. aureus ATCC 29213	4	Quality control strain for antimicrobial susceptibility testing
E. coli DH5α(pSA1)	>512	Transformant harboring a 3.5-kb BamHI fragment with *dfrG* ligated to pHSG398
E. coli DH5α(pHSG398)	<2	Transformant harboring pHSG398
E. coli DH5α(pT7dfrG)	>512	Transformant harboring PCR-amplified *dfrG* ligated to pCRT7/NT
E. coli DH5α(pT7dfrB)	128	Transformant harboring PCR-amplified intrinsic *dfrB* ligated to pCRT7/NT
E. coli DH5α(pCRT7/NT)	<2	Transformant harboring pCRT7/NT
E. coli DH5α	<2	*supE44 hsdR17 recA1 gyrA96 endA1 thi-1 relA1*

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TABLE 2. Enzyme kinetic and inhibitory properties of staphylococcal DHFRs

DHFR	Origin	K_m (μM)		IC_{50} of TMP (μM)
		DHF	NADPH	
Crude enzyme	*S. aureus* CM.S2 (IMCJ1454)	5.83 ± 2.09	15.17 ± 1.73	214
	S. aureus ATCC 25923	3.16 ± 1.99	14.78 ± 2.73	0.013
TMP-resistant DHFRs				
S3DHFR	*S. aureus* CM.S2 (IMCJ1454)	2.68 ± 1.09	2.38 ± 1.97	254
S2DHFR[a]	*S. haemolyticus* MUR313	5.1	1.7	127
S1DHFR[a]	*S. aureus*	6.6	12.4	9.8
TMP-sensitive DHFRs				
$SaDHFR_{CM.S2}$	*S. aureus* CM.S2 (IMCJ1454)	3.01 ± 1.40	2.97 ± 0.57	0.014
SaDHFR[a]	*S. aureus* ATCC 25923			0.012

[a] Data from references 6, 7, and 8.

protein is somewhat less similar to the intrinsic TMP-sensitive DHFRs from *S. aureus* (SaDHFR) (8), *S. epidermidis* (SeDHFR) (6), and *E. coli* K-12 (17), with 41%, 40%, and 40% similarity, respectively (Fig. 1). This complete ORF was named *dfrG*, and the deduced protein was designated S3DHFR. Amino acid sequence alignment of DHFRs suggests that residues involved in the binding of TMP and NADPH in other DHFRs are conserved in S3DHFR (Fig. 1). An ORF downstream of *dfrG*, designated *orfU1*, was located in the opposite direction of *dfrG* and consists of 1,950 bp encoding 650 amino acids, although the deduced amino acid sequence did not show any significant homology to sequences of other previously reported proteins. An ORF upstream of *dfrG* consisted of 582 nucleotides and was identical to the 3′-flanking region of the *SAV0404* gene encoding a hypothetical protein (11). *dfrG* and *orfU1* were flanked by a 28-bp inverted repeat and a 7-bp direct repeat,

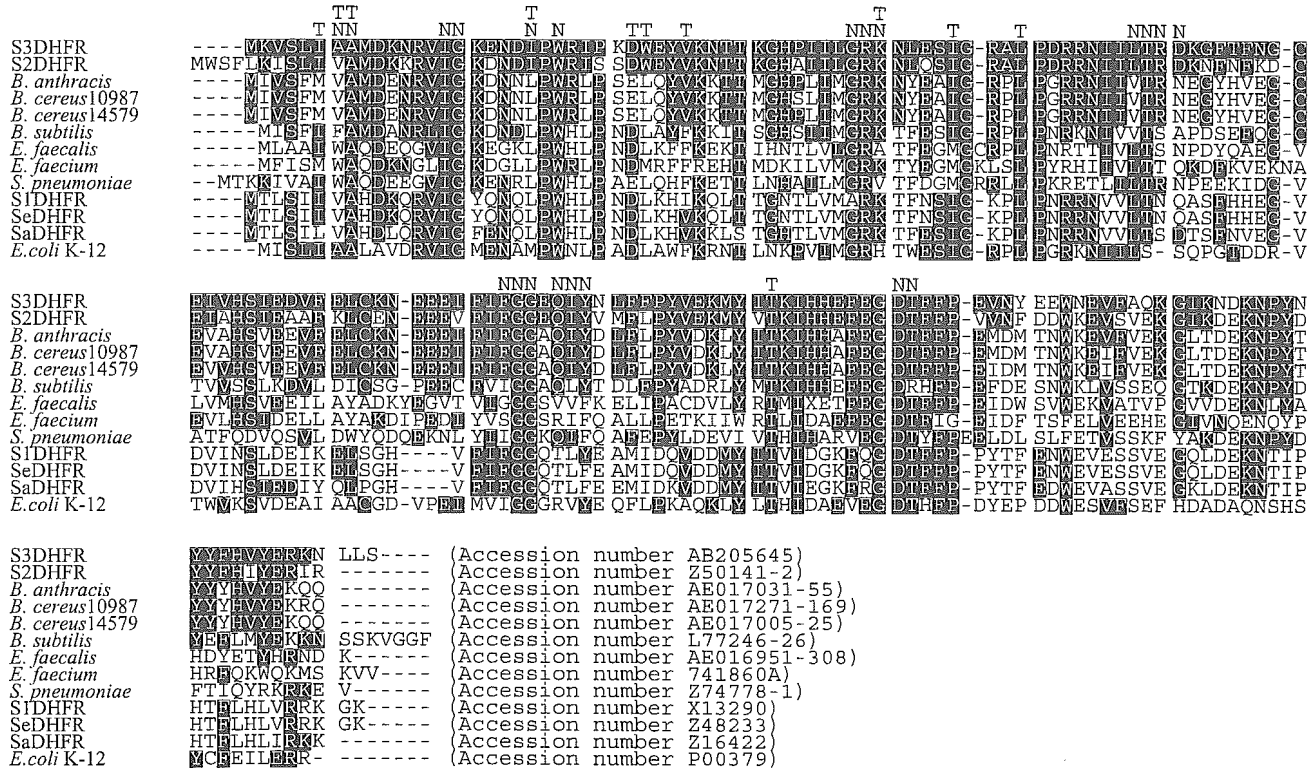

FIG. 1. Multiple-sequence alignment of the amino acid sequence of S3DHFR from *S. aureus* CM.S2 (IMCJ1454) isolate with those of DHFRs from other bacteria. The amino acid sequence of S3DHFR was compared with that of type S1 from *S. aureus*, S2 from *S. haemolyticus* MUR313, and the chromosomal DHFRs from *B. anthracis* Ames, *B. cereus* ATCC 10987, *B. cereus* ATCC 14579, *Bacillus subtilis* Marburg, *E. faecalis* V583, a methotrexate-resistant mutant of *E. faecium* strain A, *Streptococcus pneumoniae* ATCC 49619, *Staphylococcus epidermidis* ATCC 14900 (SeDHFR), *S. aureus* ATCC 25923 (SaDHFR), and *E. coli* K-12. Sequence comparison was performed by aligning the proteins with the ClustalW program (http://www.ddbj.nig.ac.jp/E-mail/clustalw-e.html). Amino acid positions involved in the binding of trimethoprim (T) and NADPH cofactor (N) are according to studies of the *E. coli* K-12 enzyme (3, 9, 13, 14). Identical residues are indicated by white letters on black background. Gaps introduced to maximize alignment are indicated by dashes.

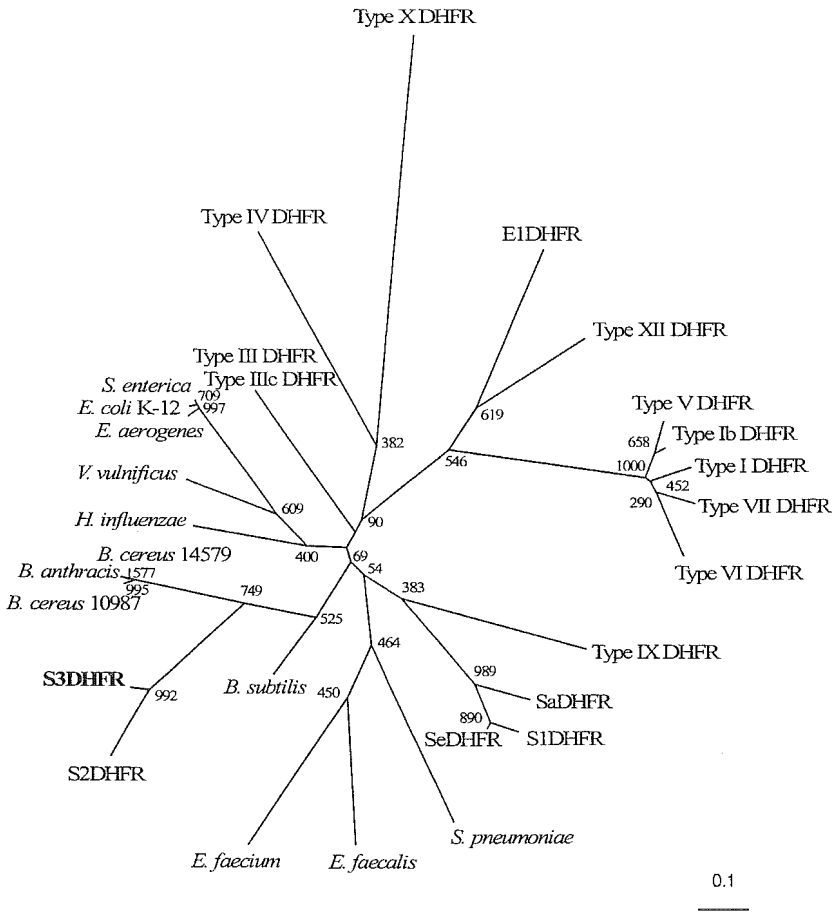

FIG. 2. Dendrogram of S3DHFR and DHFR from a variety of organisms. The dendrogram was created by the ClustalW program. Branch lengths correspond to the number of amino acid exchanges of the DHFR proteins (accession number and species given in parentheses) of types I (X00926, from *E. coli*), Ib (I40985, from *E. coli*), IV (A60935, from *E. coli*), V (X12868, from enterobacterial plasmid pLMO150), VI (Z86002, from *Proteus mirabilis*), VII (X58425, from *E. coli*), VIII (U10186, from *E. coli*), IX (A49788, from *E. coli*), X (AY123253, from *Klebsiella pneumoniae*), XII (I41043, from *E. coli*), E1 (AF028812, from *E. faecalis*), S1 (X13290, from *S. aureus*), and S2 (Z50141, from *S. haemolyticus* MUR313) and the chromosomal DHFRs of *B. anthracis* Ames (AE017031), *B. cereus* ATCC 14579 (AE017005), *B. cereus* ATCC 10987 (AE017271), *B. subtilis* Marburg (L77246), *Enterobacter aerogenes* (M26022), *E. coli* K-12 (P00379), *E. faecalis* V583 (AE016951), *E. faecium* mutant strain A (741860A), *Haemophilus influenzae* R1047 (X84205), *Salmonella enterica* serovar Paratyphi ATCC 9150 (CP000026), *S. aureus* ATCC 25923 (SaDHFR; Z16422), *S. epidermidis* ATCC 14900 (SeDHFR; Z48233), *Streptococcus pneumoniae* ATCC 49619 (Z74778), and *Vibrio vulnificus* YJ016 (BA000037).

indicating that the region is in an insertion sequence (IS). The DNA sequence, ranging from 275 bp upstream of *dfrG* to the 363 bp of the 5′-terminal region of *dfrG*, was identical to that previously reported for plasmid pMG1 in *Enterococcus faecium* (18). The *dfrG* gene may have been acquired from *E. faecium* via IS-mediated recombination. The ancestral origin of S3DHFR, however, remains unknown; S3DHFR showed little similarity to and considerable phylogenetic distance from intrinsic DHFR of *E. faecium* (Fig. 2).

The MICs of TMP in *E. coli* transformants harboring pSA1 or pT7dfrG carrying *dfrG* were significantly increased than those in control strains (Table 1), indicating that *dfrG* is responsible for TMP resistance. An *E. coli* transformant harboring pT7dfrB carrying *dfrB* also showed increased MIC, but it was not as high as those of *E. coli* strains expressing *dfrG*. *dfrB* is believed to encode a TMP-sensitive DHFR of *S. aureus* because it was found in all *S. aureus* strains, regardless of TMP susceptibility. Similar results were reported for *dfrE* encoding *Enterococcus faecalis* DHFR (4). The increased MIC for TMP in *E. coli* carrying *dfrB* may be explained by the multicopy effects of high expression of the housekeeping protein DHFR.

For functional analysis of S3DHFR and DHFR from *S. aureus* CM.S2 ($SaDHFR_{CM.S2}$), overexpression and purification of these recombinant DHFRs were achieved. Overexpression of S3DHFR or $SaDHFR_{CM.S2}$ was accomplished by integration of the respective coding regions downstream of the His-tagged coding region of the pCR/T7NT expression vector and transformation into the *E. coli* strain BL21-AI. Recombinant protein in soluble extracts was purified by affinity chromatography to determine enzymatic activities. The K_m values of recombinant S3DHFR for DHF and NADPH were 2.68 ± 1.09 μM and 2.38 ± 1.97 μM, respectively (Table 2). The K_m values of DHF and NADPH for S3DHFR did not differ from those of $SaDHFR_{CM.S2}$, but the IC_{50} values of TMP for these DHFRs differed significantly. The IC_{50} of TMP for S3DHFR was more than 8,000-fold greater than IC_{50} values for TMP-

sensitive SaDHFR and $SaDHFR_{CM.S2}$, indicating that S3DHFR and $SaDHFR_{CM.S2}$ are indeed DHFRs but that only S3DHFR plays a critical role in TMP resistance. The K_m values of crude extracts for NADPH were sixfold greater than those of recombinant S3DHFR (Table 2). Crude extracts may contain other factor(s) that bind to NADPH.

Detection of *dfrG* was performed by PCR on isolates from Chiang Mai, Thailand, and Tokyo, Japan. All Chiang Mai isolates were resistant to TMP and contained *dfrG*, whereas all Tokyo isolates but one were sensitive to TMP and did not contain *dfrG* (data not shown). The single Tokyo isolate IMCJ934 was resistant to TMP and contained *dfrG* (Table 1).

Pulsed-field gel electrophoresis (PFGE) analysis revealed 13 patterns of SmaI digestion in the 43 MRSA isolates from Chiang Mai, Thailand (data not shown). Cluster analysis showed that 12 of the 13 PFGE patterns formed a cluster (>75% similarity). The PFGE pattern of *S. aureus* CM.S2 genomic DNA was identical to that of 18 MRSA isolates. These results suggest that clonal expansion of MRSA carrying *dfrG* occurred at the hospital in Chiang Mai. The TMP-resistant isolate from Tokyo, Japan, IMCJ934, showed the same PFGE pattern as that of one of the Chiang Mai isolates, *S. aureus* CM.S2 (data not shown).

dfrG was detected by Southern blotting on fragments of SmaI-digested genomic DNA, but it was not detected on plasmids (data not shown). Conjugal transfer of TMP resistance from *S. aureus* CM.S2 to recipient strains *S. aureus* IMCJ565RFP^r or IMCJ644RFP^r was unsuccessful, suggesting that *dfrG* is located on the chromosome and not on a plasmid of these clinical isolates. It remains to be determined whether *dfrG* can be transferred by phages or mobile elements.

A single amino acid substitution (Phe to Tyr) at codon 98 of SaDHFR was reported to be associated with TMP resistance in *S. aureus* (5). Therefore, approximately 390 bp of internal DNA sequence of *dfrB* encoding SaDHFR was determined. When *S. aureus* ATCC 29213 was used as a control (5), all isolates from Chiang Mai, Thailand, exhibited three silent mutations: CAT to CAC in codon 77 and TTT to TTC in codons 91 and 118. All isolates from Tokyo, Japan, contained four silent mutations: AAA to AAG in codon 30, CAT to CAC in codon 77, and TTT to TTC in codons 91 and 118. These results indicate that these mutational changes are not associated with TMP resistance in the isolates from Chiang Mai or Tokyo. Other possible mechanisms of TMP resistance, such as overexpression of intrinsic DHFR, efflux, or impermeability, may be involved.

The CM.S2 strain was the dominant clone from Chiang Mai, Thailand. MRSA surveillance is being carried out in the hospital from which these isolates were obtained. *S. aureus* CM.S2 is resistant to clindamycin, erythromycin, gentamicin, and tetracycline and is less sensitive to arbekacin. Fosfomycin, linezolid, and vancomycin are effective in vitro; quinupristin-dalfopristin and daptomycin were not available for testing. Results of this surveillance will be reported in the future.

Our data strongly suggest that the TMP resistance-associated gene *dfrG* is prevalent in Thailand, and an isolate harboring this gene was found in Japan. This gene may spread worldwide, and measures against this, such as gene monitoring and adequate use of TMP, should be established.

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Multidrug-Resistant *Pseudomonas aeruginosa* Strain That Caused an Outbreak in a Neurosurgery Ward and Its *aac(6′)-Iae* Gene Cassette Encoding a Novel Aminoglycoside Acetyltransferase

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We characterized multidrug-resistant *Pseudomonas aeruginosa* strains isolated from patients involved in an outbreak of catheter-associated urinary tract infections that occurred in a neurosurgery ward of a hospital in Sendai, Japan. Pulsed-field gel electrophoresis of SpeI-, XbaI-, or HpaI-digested genomic DNAs from the isolates revealed that clonal expansion of a *P. aeruginosa* strain designated IMCJ2.S1 had occurred in the ward. This strain possessed broad-spectrum resistance to aminoglycosides, β-lactams, fluoroquinolones, tetracyclines, sulfonamides, and chlorhexidine. Strain IMCJ2.S1 showed a level of resistance to some kinds of disinfectants similar to that of a control strain of *P. aeruginosa*, ATCC 27853. IMCJ2.S1 contained a novel class 1 integron, In113, in the chromosome but not on a plasmid. In113 contains an array of three gene cassettes of bla_{IMP-1}, a novel aminoglycoside resistance gene, and the *aadA1* gene. The aminoglycoside resistance gene, designated *aac(6′)-Iae*, encoded a 183-amino-acid protein that shared 57.1% identity with AAC(6′)-Iq. Recombinant AAC(6′)-Iae protein showed aminoglycoside 6′-*N*-acetyltransferase activity by thin-layer chromatography. *Escherichia coli* expressing exogenous *aac(6′)-Iae* showed resistance to amikacin, dibekacin, isepamicin, kanamycin, netilmicin, sisomicin, and tobramycin but not to arbekacin, gentamicins, or streptomycin. Alterations of *gyrA* and *parC* at the amino acid sequence level were detected in IMCJ2.S1, suggesting that such mutations confer the resistance to fluoroquinolones observed for this strain. These results indicate that *P. aeruginosa* IMCJ2.S1 has developed multidrug resistance by acquiring resistance determinants, including a novel member of the *aac(6′)-I* family and mutations in drug resistance genes.

Pseudomonas aeruginosa is intrinsically resistant to many antibiotics; however, it is sensitive to a limited number of drugs, including some β-lactams, such as ceftazidime and imipenem, and aminoglycosides, such as amikacin and tobramycin. However, recent studies have shown that several strains of *P. aeruginosa* that are resistant to these antibiotics have emerged and are becoming widespread (21, 28).

In Japan, the major mechanism of resistance to aminoglycosides is production of aminoglycoside-modifying enzymes (43). The aminoglycoside 6′-*N*-acetyltransferases [AAC(6′)s] are of particular interest because they can modify a number of clinically important aminoglycosides including amikacin, gentamicin, netilmicin, and tobramycin. The AAC(6′)-I type confers resistance to amikacin through acetylation of the drug, whereas the AAC(6′)-II type acetylates gentamicin. To date, several different genes, designated *aac(6′)-Ia* to *aac(6′)-Iad*, that encode the AAC(6′)-I enzymes have been cloned and characterized (42, 50). Genes encoding aminoglycoside-modifying enzymes are often located on integrons (15), sequences that can integrate gene cassettes through site-specific recombination (17), in both plasmid and genomic DNA (15). Class 1 integrons participate in multidrug resistance in *P. aeruginosa* (27, 28, 37). Class 1 integrons contain two conserved segments (CS) that flank the antibiotic resistance gene cassettes. The 5′-CS contains the *intI1* gene, which encodes integrase, the enzyme responsible for catalysis of site-specific recombination (8). The 3′-CS contains the *qacEΔ1* and *sul1* genes and an open reading frame (ORF), orf5 (13, 16).

We describe here the genotypic and phenotypic properties of a new multidrug-resistant *P. aeruginosa* strain that caused a nosocomial outbreak of infection at a hospital in Japan. The isolate carries a class 1 integron that contains an array of three gene cassettes, including one encoding a novel aminoglycoside acetyltransferase.

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MATERIALS AND METHODS

Bacterial strains. Seven clinical isolates of *P. aeruginosa*, including *P. aeruginosa* IMCJ2.S1, were obtained from seven patients with urinary tract infections in a neurosurgery ward of a hospital in Japan. *P. aeruginosa* ATCC 27853 was obtained from the American Type Culture Collection (Manassas, Va). *Escherichia coli* strains DH5α (Takara Bio, Shiga, Japan) and BL21-AI (Invitrogen, Carlsbad, Calif.) were used as hosts for recombinant plasmids and for expression of *aac(6′)-Iae*, respectively. The rifampin-resistant *P. aeruginosa* mutant ATCC 27853 RFP^r was used. *P. aeruginosa* GN17203 (51) was provided by S. Iyobe (Kitasato University, Sagamihara, Japan).

Antibiotics and disinfectants. The antibiotics amikacin, cefoxitin, and imipenem were from Banyu Pharmaceutical Co. (Tokyo, Japan). Arbekacin and dibekacin were from Meiji Seika Kaisha (Tokyo, Japan), aztreonam was from Eizai (Tokyo, Japan), cefotaxime was from Aventis Pharma (Tokyo, Japan), and cefpodoxime and ceftazidime were from Glaxo Smith Kline (Tokyo, Japan). Cefepime was from Bristol Pharmaceuticals (Tokyo, Japan); ciprofloxacin and levofloxacin were from Daiichi Pharmaceutical (Tokyo, Japan); gentamicin, isepamicin, netilmicin, and sisomicin were from Schering-Plough (Osaka, Japan); kanamycin A and B mixture, neomycin B and C mixture, and streptomycin were from Nacalai Tesque (Kyoto, Japan); and meropenem was from Sumitomo Pharmaceutical (Osaka, Japan). Tetracycline was from Lederle Japan Co. (Tokyo, Japan); piperacillin and piperacillin-tazobactam were from Tomiyama Pure Chemical Industries (Tokyo, Japan); moxalactam, tobramycin, and sulfamethoxazole-trimethoprim were from Shionogi and Co. (Osaka, Japan); and kanamycin A, polymyxin B, and silver sulfadiazine were from Sigma Chemical (St. Louis, Mo.). The disinfectants alkyldiaminoethylglycine hydrochloride and povidone iodine were from Yoshida Pharmaceutical Co. (Tokyo, Japan); benzalkonium chloride was from Wako Pure Chemical Industries (Osaka, Japan); and chlorhexidine gluconate was from Ishimaru Pharmaceutical (Osaka, Japan).

In vitro susceptibility to antibiotics and disinfectants. MICs of antibiotics, except polymyxin B and silver sulfadiazine, were determined by the microdilution method. The MICs of polymyxin B and silver sulfadiazine were determined by the agar dilution method according to the protocols recommended by the CLSI (formerly NCCLS), standard M7-A6 (33).

Bactericidal activities of disinfectants were evaluated by time- and dose-dependent killing studies in 96-well microplates. Briefly, 10^5 microorganisms were incubated at 35°C for 0.5 min to 60 min in 160 μl disinfectants diluted serially twofold. To neutralize the bactericidal activities of the disinfectants, a 10-μl aliquot of each suspension was transferred to 200 μl Trypticase soy broth (Becton Dickinson, Franklin Lakes, NJ) containing 15% Tween 80 (Sigma), 1% soybean lecithin (Nacalai Tesque), and 0.5% sodium thiosulfate (Nacalai Tesque) and then cultured for 24 h. The minimum bactericidal concentrations (MBCs) of disinfectants were recorded relative to the duration of incubation with bacteria.

Transfer of drug resistance among bacteria. Transfer of the drug resistance from *P. aeruginosa* clinical isolates to a rifampin-resistant mutant of *P. aeruginosa*, ATCC 27853 RFP^r, was examined with the broth mating method (25). After mating, transconjugants were selected on Mueller-Hinton agar plates containing rifampin (200 μg/ml) and imipenem (16 μg/ml) or amikacin (20 μg/ml). Plasmid DNAs from the clinical isolates were purified either with a QIAprep kit (QIAGEN, Tokyo, Japan), by Kado and Liu's (24), or method by the method of Domenico et al. (11). With the QIAprep kit or Kado and Liu's method, the bacteria were lysed at different temperatures, 22°C for 5 min or 60°C for 70 min for each method.

PCR of class 1 integrons. To identify the presence of a class 1 integron and to determine the size of any inserted gene cassettes, PCR amplification was performed as described previously (29) with primers 5′-cs and 3′-cs, which are specific for 5′-CS and the 3′-CS of class 1 integrons, respectively, and an Expand High Fidelity PCR system (Roche Diagnostics GmbH, Penzberg, Germany). To determine the content and order of genes in the integron, PCR amplification of the variable region of class 1 integrons was carried out with the primers listed in Table 1. All PCRs were performed with a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems, Foster City, Calif.). Genomic DNAs extracted as described by Sambrook and Russell (41) were used as templates. Amplification conditions were 30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 3 min or 5 min. PCR for amplicons longer than 1 kb was performed with 1.25 U of Z-*Taq* polymerase (Takara Bio) and 30 cycles of 95°C for 1 s and 68°C for 120 s according to the manufacturer's instructions.

PCR of QRDRs. The *gyrA*, *gyrB*, *parC*, and *parE* quinolone resistance-determining regions (QRDRs) of *P. aeruginosa* were amplified by PCR with the primers listed in Table 1 according to methods described previously (1, 21, 26, 31). PCR products were sequenced with the same primers.

DNA sequencing. DNA sequences were determined by the dideoxy chain termination method with an ABI PRISM 3100 sequencer (Applied Biosystems). Homology searches of nucleotide and deduced protein sequences were performed by FASTA and BLAST screens of the DDBJ, GenBank, and EMBL databases. Multiple-sequence alignments and searches for ORFs were performed with GENETYX-WIN software (Genetyx, Tokyo, Japan). The dendrogram for AACs was calculated with the CLUSTAL W Program (49).

Cloning of the *aac(6′)-Iae* gene. The coding region of *aac(6′)-Iae* (Fig. 1) was amplified by PCR with 2.5 U of *Ex Taq* DNA polymerase (Takara Bio) and primers aacS1-FC and aacS1-RC (Table 1). The PCR products were cloned into pCRT7/NT (Invitrogen) downstream of the region encoding a six-His tag. Then plasmid pAAC6, which contains *aac(6′)-Iae*, or plasmid pREVAAC6, which contains *aac(6′)-Iae* in the reverse direction, was transformed into *E. coli* DH5α cells by the $CaCl_2$ method (6). DNA sequences of these cloned fragments were verified by sequencing of both strands as described above.

Purification of recombinant AAC(6′)-Iae. *E. coli* BL21-AI harboring plasmid pAAC6 was grown to an A_{600} of 0.2 to 0.3 in LB medium containing 50 mg/liter ampicillin at 37°C. After addition of arabinose (final concentration, 0.02%) to induce expression of AAC(6′)-Iae, the *E. coli* strain was cultured for another 18 h at 25°C. The bacterial cells were collected, resuspended in 50 mM HEPES buffer (pH 7.5) containing 0.1% Triton X-100, and lysed by sonication on ice for 15 s 40 times and then for 20 s 100 times. After centrifugation to remove the debris, the solubilized protein was applied to an AKTA Prime (Amersham Biosciences, Piscataway, NJ) system equipped with a HiTrap Chelating HP column (Amersham Biosciences) loaded with Ni^{2+}. The column was washed with 20 mM Tris-HCl (pH 7.9) containing 60 mM imidazole and 0.5 M NaCl and was eluted with the same buffer containing 1 M imidazole. The eluted proteins were collected and dialyzed in 50 mM HEPES buffer (pH 7.5). The protein preparation yielded a single band upon sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis (data not shown).

Acetylation of aminoglycosides by recombinant ACC(6′)-Iae. Enzymatic acetylation of aminoglycosides was done as described previously (53). Recombinant AAC(6′) from actinomycete strain #8 was provided by J. Ishikawa (National Institute of Infectious Diseases, Tokyo, Japan). Various aminoglycosides were incubated with recombinant AAC(6′)-Iae or AAC(6′) as a positive control in the presence of acetyl coenzyme A, and the acetylated derivatives were detected by thin-layer chromatography. The reaction was carried out at 37°C for 30 min to 12 h.

Pulsed-field gel electrophoresis (PFGE). Genomic DNA from *P. aeruginosa* was prepared by the procedure of Grundmann et al. (14) and digested overnight with 10 U of SpeI, XbaI, or HpaI (Takara Bio). The DNA fragments were separated on 1.0% agarose gels in 0.5× Tris-borate-EDTA buffer with a CHEF Mapper system (Bio-Rad Laboratories, Hercules, Calif.) at 6 V/cm for 20 h.

Southern hybridization. We performed Southern blotting to identify the location of In113. A 465-bp segment of *aac(6′)-Iae* and a 362-bp segment of bla_{IMP-1} amplified by PCR were labeled with horseradish peroxidase and used as probes.

Nucleotide sequence accession number. The nucleotide sequence of In113 reported here has been deposited in the EMBL/GenBank/DDBJ databases and assigned accession number AB104852.

RESULTS

Epidemiologic analysis of a nosocomial outbreak of *P. aeruginosa*. From June 2002 to November 2002, a *P. aeruginosa* outbreak occurred in a neurosurgery ward of a 500-bed hospital in Japan. Three patients developed catheter-associated urinary tract infections with multidrug-resistant *P. aeruginosa* in June 2002. Various measures for infection control were undertaken, but four patients subsequently developed similar catheter-associated urinary tract infections with multidrug-resistant *P. aeruginosa* over the next 5 months. Seven *P. aeruginosa* isolates from these patients were analyzed by PFGE. The PFGE patterns of SpeI-, XbaI-, or HpaI-digested genomic DNAs from the isolates were identical, indicating that the isolates were all from monoclonal expansion of a single multidrug-resistant *P. aeruginosa* strain. This clone was named *P. aeruginosa* IMCJ2.S1. PFGE patterns of SpeI-, XbaI-, and

TABLE 1. PCR primers

Primer	Sequence[a] (5′→3′)	Expected size of amplicon (bp)	Position (nt)[b]	Reference or GenBank accession no.
5′-cs	GGCATCCAAGCAGCAAG			29
3′-cs	AAGCAGACTTGACCTGA			29
int1-F	TGCGTGTAAATCATCGTCGT	838	Downstream of *intI1*	AF071413
int1-R	CGAAGTCGAGGCATTTCTGT		177–196 in *intI1*	AF071413
IMP-F[c]	DTTYCTAAACAYGGYTTGGT	362	145–164 in bla_{IMP-1}	AB070224
IMP-R[c]	YTTTYAGGYARCCAAACYACT		486–506 in bla_{IMP-1}	AB070224
aacS1-F	CGCAAGCTGCAGAAATTCTAT	465	47–67 in *aac(6′)-Iae*	This study
aacS1-R	TCCCATTTGCATTAGGAATCA		491–511 in *aac(6′)-Iae*	This study
aadA1-F	TGATTTGCTGGTTACGGTGA	451	144–163 in *aadA1*	AF071413
aadA1-R	TACTGCGCTGTACCAAATGC		575–594 in *aadA1*	AF071413
qacEdelta-F	TGAAAGGCTGGCTTTTTCTT	286	2–21 in *qacEΔ1*	AF071413
qacEdelta-R	GCAATTATGAGCCCCATACC		268–287 in *qacEΔ1*	AF071413
sul-F	TCACCGAGGACTCCTTCTTC	759	29–48 in *sul1*	AF071413
sul-R	GGGTTTCCGAGAAGGTGATT		768–787 in *sul1*	AF071413
int1imp1-F	AGCACCTTGCCGTAGAAGAA	695	262–281 in *intI1*	AJ640197
int1imp1-R	TTTTATAGCCACGCTCCACA		243–262 in bla_{IMP-1}	AJ640197
imp1aacS1-F	AAAGGCAGCATTTCCTCTCA	737	265–284 in bla_{IMP-1}	This study
imp1aacS1-R	GACGGCCAAGAATCGAAAT		89–107 in *aac(6′)-Iae*	This study
aacS1aadA1-F	ATTGTGTGGTTGGGTTGGAT	691	186–205 in *aac(6′)-Iae*	This study
aacS1aadA1-R	GGAGAATCTCGCTCTCTCCA		231–259 in *aadA1*	This study
aadA1qacEd-F	TGATTTGCTGGTTACGGTGA	873	144–163 in *aadA1*	AF071413
aadA1qacEd-R	ATGCGGATGTTGCGATTACT		42–61 in *qacEΔ1*	AF071413
qacEdsul-F	TCGGTGTTGCTTATGCAGTC	306	167–186 in *qacEΔ1*	AF071413
qacEdsul-R	ACATCCACGACGTCTGATCC		112–131 in *sul1*	AF071413
int-R	TGCGTGTAAATCATCGTCGT	3,172	Downstream of *intI1*	AF071413
sul-R	GGGTTTCCGAGAAGGTGATT		768–787 in *sul1*	AF071413
sul-F	TCACCGAGGACTCCTTCTTC	6,474	29–48 in *sul1*	AF071413
tniB-R	ATCATCGACCTGTCCCACCT		16–35 in *tniBΔ1*	AF071413
tniB-F	CAGAGCCAGTTGCTCCATTT	1,749	395–414 in *tniBΔ1*	AF071413
tniA-R	CTTTCACCGCGAAGTCACTC		384–403 in *tniA*	AF071413
GyrA1	TTATGCCATGAGCGAGCTGGGCAACGACT	366	147–176 in *gyrA*	26
GyrA2	AACCGTTGACCAGCAGGTTGGGAATCTT		484–512 in *gyrA*	26
GyrB1	GCGCGTGAGATGACCCGCCGT	390	1162–1182 in *gyrB*	31
GyrB2	CTGGCGGTAGAAGAAGGTCAT		1531–1551 in *gyrB*	31
PARC1	ATGAGCGAACTGGGGCTGGAT	210	166–187 in *parC*	21
PARC2	ATGGCGGCGAAGGACTTGGGA		354–375 in *parC*	21
ParE1	CGGCGTTCGTCTCGGGCGTGGTGAAGGA	592	1223–1250 in *parE*	1
ParE2	TCGAGGGCGTAGTAGATGTCCTTGCCGA		1787–1814 in *parE*	1
aacS1-FC	ATGAAATACAACATTGTTAATATTA	552	1–25 in *aac(6′)-Iae*	This study
aacS1-RC	TTACATTATATTTTTCCACATTAAT		528–552 in *aac(6′)-Iae*	This study

[a] D stands for adenine, thymine, or guanine; R stands for adenine or guanine; Y stands for cytosine or thymine.
[b] Nucleotides are numbered according to deposited sequences.
[c] Primer designed to amplify bla_{IMP-1} (accession no. AB070224) or homologous genes, including bla_{IMP-2} (AJ243491), bla_{IMP-3} (AB010417), bla_{IMP-4} (AF445082), bla_{IMP-5} (AF290912), bla_{IMP-6} (AB040994), bla_{IMP-7} (AF416736), bla_{IMP-8} (AF322577), bla_{IMP-9} (AY033653), bla_{IMP-10} (AB074434), and bla_{IMP-11} (AB074437).

HpaI-digested genomic DNAs from IMCJ2.S1 are shown in Fig. 2A.

Susceptibility of *P. aeruginosa* IMCJ2.S1 to antibiotics and disinfectants. The MICs of various antibiotics, including potent active β-lactams, against IMCJ2.S1 were compared with those against a reference strain, *P. aeruginosa* ATCC 27853 (Table 2). IMCJ2.S1 was resistant to all antibiotics tested except for arbekacin and polymyxin B. Strain ATCC 27853 was sensitive to all of the antibiotics tested except cefoxitin, flomoxef, and kanamycin. Thus, IMCJ2.S1 was classified as a multidrug-resistant strain of *P. aeruginosa*.

To test whether IMCJ2.S1 showed increased resistance to disinfectants, the MBCs of four disinfectants, povidone iodine, alkyldiaminoethylglycine hydrochloride, benzalkonium chloride, and chlorhexidine gluconate, were determined for both IMCJ2.S1 and ATCC 27853. Both strains were resistant to chlorhexidine gluconate but sensitive to povidone iodine (MBC, <0.001% [wt/vol]), alkyldiaminoethylglycine hydrochloride (MBC, <0.001% [wt/vol]), and benzalkonium choride (MBC, <0.005% [wt/vol]). The MBC patterns of these strains were identical. These results indicate that the sensitivity of IMCJ2.S1 to disinfectants is not different from that of the *P. aeruginosa* reference strain.

Detection of an integron in *P. aeruginosa* IMCJ2.S1. To determine if strain IMCJ2.S1 carried a class 1 integron, PCR analysis specific for class 1 integrons was performed (29). Strain IMCJ2.S1 yielded a 2.5-kbp PCR product, whereas *E. coli* CSH2 harboring plasmid NR1 (32), which carries In2 (30), yielded a 1.0-kbp PCR product. *P. aeruginosa* ATCC 27853 did not yield PCR products. These results suggest that strain IMCJ2.S1 and *E. coli* CSH2 each carry a class 1 integron and that this integron contains additional sequences that are not present in In2.

The class 1 integron frequently contains the *tniB* and *tniA* genes downstream of the 3′-CS (13, 16). To confirm the presence of a class 1 integron in IMCJ2.S1 and to elucidate the

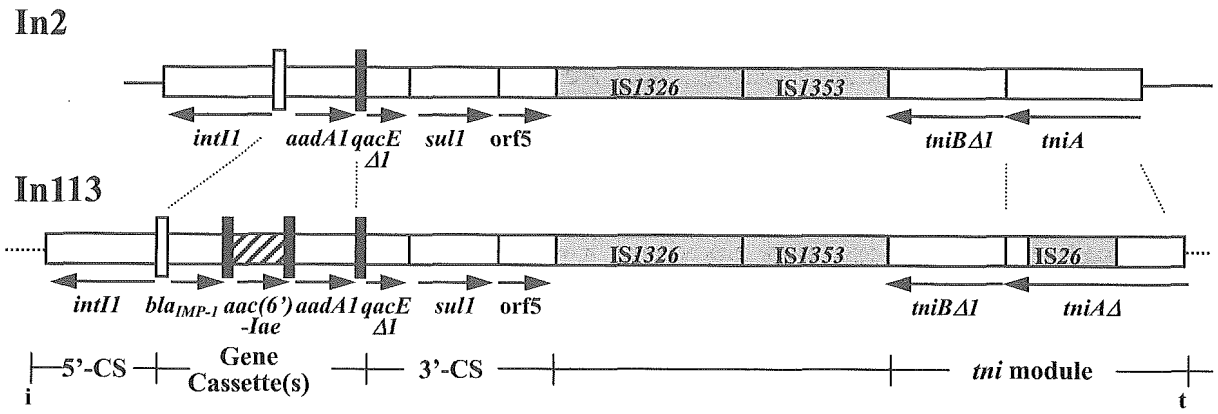

FIG. 1. Structure of In2 (GenBank accession no. AF071413) and In113. Gene cassettes are represented as open boxes with an adjacent vertical bar (59-be), shown as heavy solid vertical bars. The novel ORF found in In113 is shown as a hatched box. Genes are indicated by horizontal arrows. IS are represented as gray boxes and are labeled. The sites of the 5′-CS, gene cassettes, 3′-CS, and *tni* module are indicated just below the construct. IRi and IRt are shown as vertical lines labeled i and t, respectively, and the *attI1* sites are shown as open vertical bars toward the left of the constructs.

structure downstream of the 3′-CS, we performed PCR specific for *intI1*, *qacEΔ1*, *sul1*, and their spanning or marginal regions. PCRs yielded the expected products (Table 1), with the exception of a 4.7-kbp fragment after amplification with int-R and sul-R and a 2.5-kbp fragment after amplification with tniB-F and tniA-R. These data show that IMCJ2.S1 carries a class 1 integron and that this integron contains *intI1-sul1* in a 4.7-kbp region, *sul1-tniB* in a 6.5-kbp region, and *tniB-tniA* in a 2.5-kbp region (Fig. 1).

Identical results were obtained for the other six isolates from the outbreak.

Structure of the class 1 integron found in *P. aeruginosa* IMCJ2.S1. We analyzed the sequences of the PCR products to determine the structure of the class 1 integron of IMCJ2.S1. The 5′-CS contained *intI1*, the *attI1* recombination site with a 7-bp core site sequence of GTTAGAA (45), and the TGGACA (−35) and TAAACT (−10) hexamers separated by 17 bp, which is characteristic of the Pc promoter (7, 45). Although TTGTTA (−35) and TACAGT (−10) hexamers separated by 14 bp were present again downstream of the Pc promoter, this region is not likely to act as the P2 promoter, because there is no GGG sequence (7, 45).

Between the 5′-CS and 3′-CS, there were three gene cassettes (Fig. 1). The 880-nucleotide (nt) cassette contained the metallo-β-lactamase gene bla_{IMP-1} (35) and a 127-nt 59-base element (59-be) site, a site for site-specific cointegration events (Fig. 3), and this cassette was identical to one described previously (2, 35). The 647-nt cassette contained an ORF and a 68-nt 59-be site (Fig. 3). The sequence of this 647-nt cassette was not found in any database, and therefore, we named this integron In113 (Fig. 1). The ORF in the 647-nt cassette encoded a 183-amino-acid (aa) product that was 55.2% identical to a 6′-*N*-aminoglycoside acetyltransferase, AAC(6′)-Ia (48), and 57.1% identical to AAC(6′)-Iq of *Klebsiella pneumoniae* (4). We named the predicted protein AAC(6′)-Iae according to the standard nomenclature (42).

AAC(6′)-Iae was relatively similar to a subfamily of AAC(6′)-I enzymes that includes AAC(6′)-Ia (48), AAC(6′)-Iq (4), and AAC(6′)-Im (19) [which is not the AAC(6′)-Im reported by Chow et al. (5) and has also been

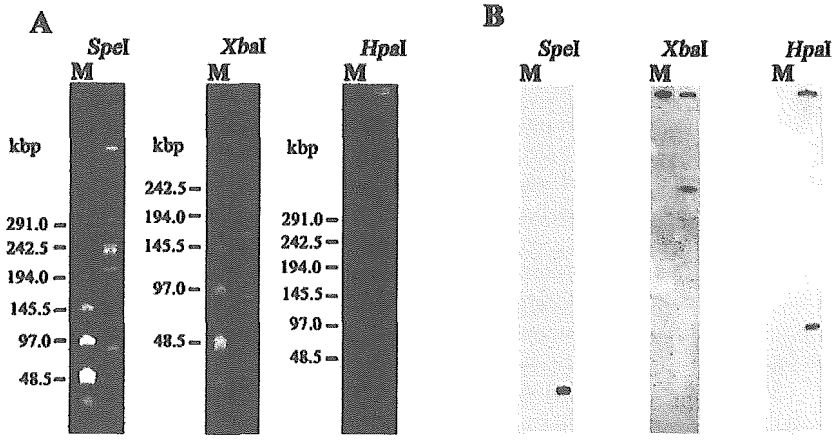

FIG. 2. (A) PFGE of SpeI-, XbaI-, and HpaI-digested genomic DNA from multidrug-resistant *P. aeruginosa* IMCJ2.S1. (B) Southern blotting of the same gels with an *aac(6′)-Iae* probe. Lanes M, HindIII-digested λ phage DNA as a size marker.